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MAGI-1 acts as a scaffolding molecule for NGF receptor-mediated signaling pathway

Hidenori Ito, Rika Morishita, Ikuko Iwamoto, Makoto Mizuno, Koh-ichi Nagata *

Department of Molecular Neurobiology, Institute for Developmental Research, Aichi Human Service Center, Kasugai, Aichi, Japan

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ABSTRACT

We have recently found that the membrane-associated guanylate kinase with inverted organization-1 (MAGI-1) was enriched in rat nervous tissues such as the glomeruli in olfactory bulb of adult rats and dorsal root entry zone in spinal cord of embryonic rats. In addition, we revealed the localization of MAGI-1 in the growth cone of the primary cultured rat dorsal root ganglion cells. These results point out the possibility that MAGI-1 is involved in the regulation of neurite extension or guidance. In this study, we attempted to reveal the physiological role(s) of MAGI-1 in neurite extension. We found that RNA interference (RNAi)-mediated knockdown of MAGI-1 caused inhibition of nerve growth factor (NGF)-induced neurite outgrowth in PC12 rat pheochromocytoma cells. To clarify the involvement of MAGI-1 in NGF-mediated signal pathway, we tried to identify binding partners for MAGI-1 and identified p75 neurotrophin receptor (p75NTR), a low affinity NGF receptor, and Shc, a phosphotyrosine-binding adaptor. These three proteins formed an immunocomplex in PC12 cells. Knockdown as well as overexpression of MAGI-1 caused suppression of NGF-stimulated activation of the Shc-ERK pathway, which is supposed to play important roles in neurite outgrowth of PC12 cells. These results indicate that MAGI-1 may act as a scaffolding molecule for NGF receptor-mediated signaling pathway.

1. Introduction

The membrane-associated guanylate kinase with inverted organization (MAGI) proteins belong to a subfamily of membrane-associated guanylate kinase (MAGUK) proteins, and consist of three members, MAGI-1, MAGI-2/synaptic scaffolding molecule (S-SCAM) and MAGI-3. They usually harbor six PSD-95/Discs Large/Zona Occludens (PDZ) domains, a GUK domain and two WW motifs [1–3]. MAGI-2/S-SCAM is the neural isoform of MAGI protein and interacts with various synaptic molecules such as NMDA receptors, neuroligins and AMPA receptors [2,4,5]. Physiological functions of MAGI-1 have been investigated mainly in non-neuronal tissues; it is localized at tight junction of epithelial cells [6] and cell–cell contacts of vascular endothelial cells [7]. Although MAGI-1 is thought to act as a scaffold for various proteins such as β-catenin, RapGEP and JAM4 at non-neuronal cell junctions [8–10], the physiological significance of MAGI-1 in neuronal tissues is yet obscure. We have recently developed the specific antibody for MAGI-1 and found that MAGI-1 was enriched in rat neural tissues such as the glomeruli in olfactory bulb of adult rats and dorsal root entry zone in spinal cord of embryonic rats. In addition, we found the localization of MAGI-1 in the growth cone of primary cultured rat dorsal root ganglion neurons [11]. These results appeared to indicate the possible participation of MAGI-1 in neurite extension or guidance.

Neurotrophins are a family of proteins that influence various activities of neuronal cells such as proliferation, growth and differentiation [12,13]. They interact with two distinct classes of receptors, tropomyosin-related kinase (Trk) family consisting of TrkA, TrkB and TrkC, and p75 neurotrophin receptor (p75NTR). Four neurotrophins, NGF, BDNF, NT-3 and NT-4, exhibit specificity for Trk receptors, and NGF interacts only with TrkA [14]. The cytoplasmic domain of Trk can interact with various molecules involved in signaling cascade such as Ras-extracellular signal-regulated kinase (ERK), phosphatidylinositol-3 (PI3) kinase and phopholipase C (PLC)-γ1 signaling. In PC12 rat pheochromocytoma cells, NGF binds to TrkA with high affinity and induces Ras-mediated activation of ERK that leads to subsequent proliferation and differentiation [12]. On the other hand, p75NTR does not possess a cytoplasmic tyrosine kinase domain and interacts with Trk receptors. As a Trk co-receptors p75NTR binds each of neurotrophins with relatively low affinity [15], and has been shown to enhance Trk receptor activity mediated by neurotrophins [16,17]. While p75NTR does not have intrinsic catalytic activity, this protein interacts with diverse signaling molecules such as tumor necrosis factor receptor-associated factor-6 (TRAF6), RhoA

Abbreviations: NGF, nerve growth factor; MAGI-1, membrane-associated guanylate kinase with inverted organization-1; MAGUK, membrane-associated guanylate kinase; PDZ, PSD-95/Discs Large/Zona Occludens; Trk, tropomyosin-related kinase; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; FBS, fetal bovine serum; PBS, phosphate-buffered saline; GST, glutathione S-transferase; MBP, maltose binding protein; IP, immunoprecipitation; PAGE, polyacrylamide gel electrophoresis; RNAi, RNA interference; PTB, phosphotyrosine binding; JNK, c-Jun N-terminal kinase

* Corresponding author at: Department of Molecular Neurobiology, Institute for Developmental Research, Aichi Human Service Center, 713-8 Kamiya, Kasugai, Aichi 480-0392, Japan. Tel.: +81 568 88 0811x3585; fax: +81 568 88 0829.
E-mail address: knagata@inst-hsc.jp (K. Nagata).

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and Nogo receptors and exerts diverse signaling pathways leading to apoptosis, cell survival and neurite outgrowth [18–20].

Here, we demonstrate that MAGI-1 regulates NGF-stimulated neurite extension of PC12 cells. Moreover we found that MAGI-1 interacts with p75NTR and Shc, and acts as a scaffolding molecule for NGF signaling pathway.

2. Materials and methods

2.1. Plasmid construction

pRK5-myc-mouse MAGI-1 was obtained from Dr. K. Patrie (University of Michigan). Mouse MAGI-2 and MAGI-3 were from Drs. Y. Hata (Tokyo Medical and Dental University, Japan) and E. Peles (The Weizmann Institute of Science, Israel), respectively. Full length and various fragments of MAGI proteins, p75NTR and Shc were produced by PCR and subcloned into pCAG-GFP-MCS2, pCAG-myc-MCS2, pCAG-FLAG-MCS2 [21], EGFP-C1(Clontech Laboratories, Inc., CA, USA), pMal (New England Biolab. Inc., MA) and pGEX-4T3 (GE Healthcare Bio-Sciences, Uppsala, Sweden) vectors. All constructs were verified with DNA sequencing. GFP-ERK was obtained from Dr. N. Kioka (Kyoto University, Japan).

2.2. Antibodies and reagents

Using glutathione S-transferase (GST)-fused p75NTR fragment (p75-intracellular domain, aa 274–472) expressed in *Escherichia coli* as an antigen, a rabbit polyclonal antibody for p75NTR, anti-p75, was generated and affinity-purified on a column to which the antigen had been conjugated. A MAGI-1-specific antibody, anti-MAGI-1, and pan-MAGI antibody, anti-MAGI1/2/3, were prepared as described previously [11]. Polyclonal rabbit anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr/204), anti-Shc, anti-phospho Shc (Tyr239/240) (Cell Signaling Technology Inc., Danvers, MA, USA), and anti-GFP (Medical & Biological Laboratories Co., Nagoya, Japan) were purchased. Rat monoclonal anti-GFP was obtained from Nacalai Tesque (Kyoto, Japan). Monoclonal mouse anti-p75NTR (clone 192-IgG, Millipore), anti-GFP (Santa Cruz Biotech. Inc., Santa Cruz, CA, USA) and anti-myc 9E10 [22] were also used.

2.3. Cell culture and transfection

COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. PC12 cells were cultured in DMEM supplemented with 10% horse serum, 5% FBS and antibiotics. Transient transfection was carried out with the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

2.4. Immunofluorescent analysis

Immunofluorescent analysis was done as described [23]. Alexa Fluor® 488 and 568 (Invitrogen) were used as secondary antibodies. Fluorescent images were obtained using FV-1000 confocal microscope (Olympus Corporation, Tokyo, Japan).

2.5. RNA interference

The following target sequences were inserted to pSUPER-puro or pSUPER-GFP vector (OligoEngine, Seattle, WA, USA), rat MAGI-1; 5′-GTCGGCACCTATGAAGGAA-3′ (534–552, MAGI#1), 5′-AGTGATCACGAC GGATGCC-3′ (599–617, MAGI#2). Numbers indicate positions from transcription start sites. We usually used pSUPER-puro vector for biochemical analysis and pSUPER-GFP vector for cell biological analysis. To generate RNAi-resistant mutants of MAGI-1, MAGI-1#1mt and MAGI-1#2mt, we introduced three silent mutations, as underlined, in the target sequences of pSUPER-MAGI-1#1 (5′-GTCGGGACCTACGAG GGAA-3′) and pSUPER-MAGI-1#2 (5′-AGTCATCACCACCGATGCC-3′).

2.6. Quantification of neurite-bearing cells

PC12 cells were transfected with various vectors and cultured for 48 h. Then, neurite extension was induced by DMEM with 1% horse serum, 0.5% FBS and 100 ng/ml NGF for 48 to 72 h. Cells were fixed and stained as described. Images were obtained using a confocal microscope. Morphometric measurements were performed using ImagePro PLUS software (Media Cybernetics, Silver Spring, MD, USA). For quantification of neurite bearing cells, 100–150 cells were randomly chosen and length of processes was measured. Cells with a process longer than one cell diameter were defined as neurite-bearing cells. Statistical analyses were performed by the Student's *t*-test.

2.7. Immunoprecipitation

Immunoprecipitation was done as previously described [24]. Briefly, cells expressing tagged proteins were harvested with immunoprecipitation (IP) buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 1 mM Na_3VO_4 and protease inhibitor cocktail (Nacalai Tesque). Insoluble material was removed by centrifugation at 4 °C for 10 min at 10,000 ×*g* and the resultant supernatants were subjected to immunoprecipitation using indicated antibody and proteinA-sepharose beads. After washing the beads with IP buffer, the precipitates were subjected to SDS-PAGE followed by western blotting. Immunoreactive bands were visualized as described [25]. In the experiment of the binding of MAGI-1 and Shc, we used IP buffer without NaCl. To determine the complex formation in PC12 cells, cells were lysed in a buffer consisting of Tris–buffered saline (pH 8.0) with 0.1% Triton X-100, 60 mM octylglucoside, 1 mM Na_3VO_4 and protease inhibitor cocktail.

2.8. In vitro binding assay

Full length MAGI-1 was constructed into pMal vector. The fusion protein was then purified by one-step affinity purification specific for maltose-binding protein (MBP) according to the manufacturer's instruction. GST-fused proteins were immobilized on to glutathione–sepharose 4B (GE Healthcare Bioscience) and incubated with MBP or MBP-fused MAGI-1 in IP buffer for 1 h at 4 °C. The beads were then washed with IP buffer and bound proteins were analyzed by western blotting.

2.9. Analyses of ERK and Shc activation

PC12 cells were transfected with GFP-ERK or GFP-Shc in the presence or absence of a MAGI-1 knockdown vector and cultured for 48 h. Cells were then serum-starved for 24 h and stimulated by 100 ng/ml NGF for the indicated time periods. Cells were fixed with 10% trichloroacetic acid, washed with PBS, suspended in lysis buffer (50 mM Tris–HCl buffer, pH 7.5, containing 0.1 M NaF, 5 mM EDTA, 2% SDS and protease inhibitor) and centrifuged at 10,000 ×*g* for 10 min. Cleared supernatants were subjected to SDS-PAGE followed by western blot analyses using anti-phospho p44/p42 MAP kinase, anti-phospho Shc or anti-GFP. Relative intensities of bands were quantified using ImagePro PLUS software. Statistical analyses were performed by the Student's *t*-test.

3. Results

3.1. Knockdown of MAGI-1 caused the suppression of NGF-induced neurite outgrowth in PC12 cells

Based on our pervious study [11], we explored possible role of MAGI-1 in neurite extension. Effect of MAGI-1 knockdown on the NGF-induced neurite extension was examined in PC12 rat pheochromocytoma cells since this cell line is widely used to study the mechanism of neurite extension [26]. We constructed two RNAi vectors,

pSUPER-MAGI-1#1 and #2, containing different target sequences in rat MAGI-1 and estimated the knockdown efficiency to exogenously expressed mouse MAGI-1 in COS7 cells because these target sequences were conserved in both species. As shown in Fig. 1A, expression of GFP-MAGI-1 was reduced by these RNAi vectors. We next generated RNAi-resistant versions of MAGI-1, pEGFP-MAGI#1mt and -MAGI#2mt, that harbor silent mutations within the sequences targeted by pSUPER-MAGI-1#1 and #2, respectively. Western blot analyses confirmed the resistance of MAGI#1mt and MAGI#2mt to pSUPER-MAGI-1#1 and #2, respectively (Fig. 1A).

Then we tried to confirm the effect of these RNAi vectors against endogenous MAGI-1 in PC12 cells. We however could not observe the significant reduction of endogenous MAGI-1 in western blotting, probably due to the low transfection efficiency; when PC12 cells were transfected with pSUPER-GFP vector, the efficiency was ~10% based on GFP detection in immunofluorescence (data not shown). We thus performed immunofluorescence analyses, and knockdown of endogenous MAGI1 was observed as shown in Fig. 1B. We next analyzed possible effect of MAGI-1-knockdown on NGF-induced neurite extension, and found that the extension was significantly impaired in MAGI-1-deficient PC12 cells (Fig. 1C and D). It is notable that NGF-induced neurite extension was also impaired when MAGI-1 was overexpressed in the cell (Fig. 1E and F). These results suggest that MAGI-1 plays pivotal roles in NGF-stimulated neurite outgrowth in PC12 cells.

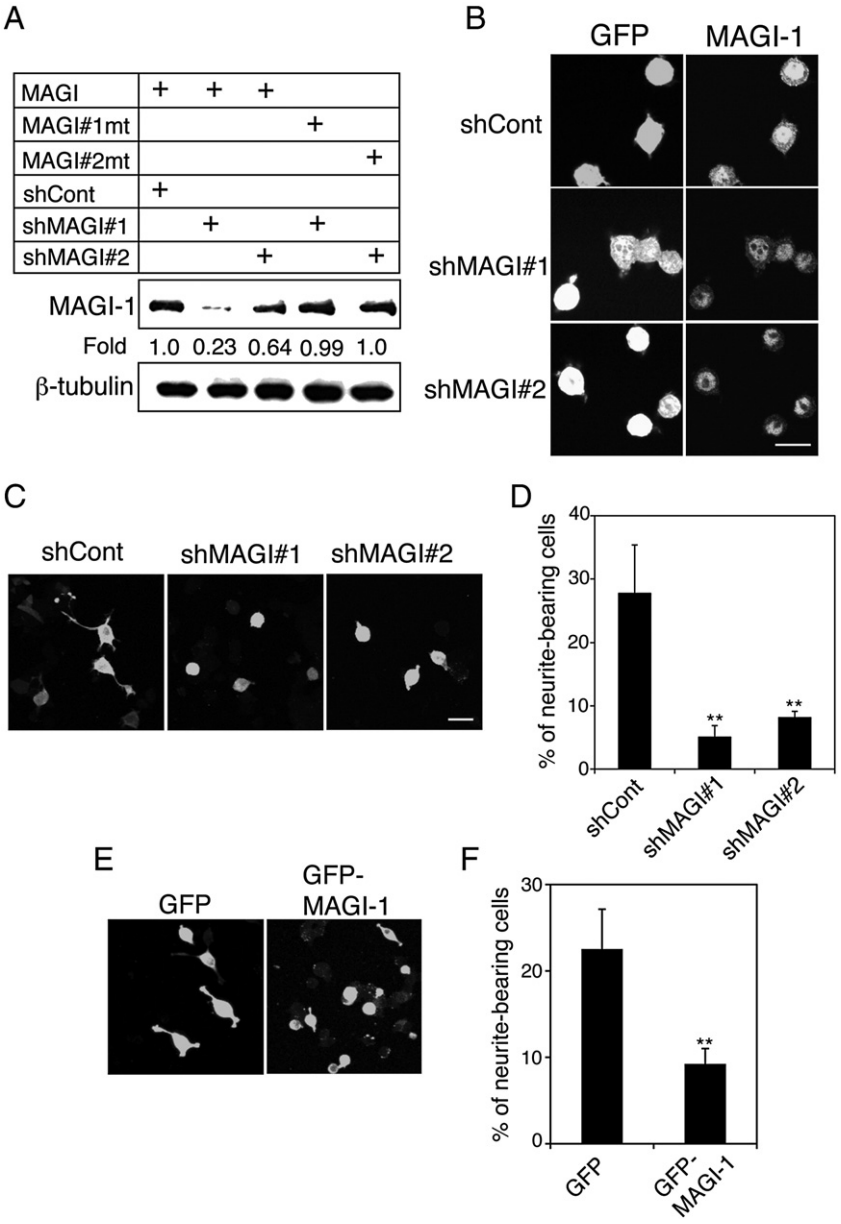

Fig. 1. Knockdown or overexpression of MAGI-1 caused inhibition of NGF-dependent neurite outgrowth in PC12 cells. (A) COS7 cells were transfected with pEGFP-MAGI-1, pEGFP-MAGI#1mt, pEGFP-MAGI#2mt, pSUPER (shCont), pSUPER-MAGI#1 (shMAGI#1) or pSUPER-MAGI#2 (shMAGI#2) in various combinations. After 48 h, cells were harvested and the lysates (10 μg of protein) were subjected to western blotting using anti-GFP. The expression of β-tubulin was examined to confirm the equal loading. (B) PC12 cells were transfected with control pSUPER-GFP, pSUPER-GFP-MAGI#1 (shMAGI#1) or -MAGI#2 (shMAGI#2). After 48 h, cells were fixed and stained with monoclonal anti-GFP and polyclonal anti-MAGI-1. (C) PC12 cells were transfected with pSUPER-GFP vector (*left panel*), pSUPER-GFP-MAGI#1 (*middle panel*) or pSUPER-GFP-MAGI#2 (*right panel*). After 48 h, medium was changed to NGF-containing serum reduced medium and cultured for additional 48 h. Then cells were fixed and stained with anti-GFP. (D) The percentage of neurite-bearing cells in (C) was calculated and graphed. $^{**}p < 0.01$. (E) PC12 cells were transfected with pEGFP vector (*left panel*) or pEGFP-MAGI-1 (*right panel*). Analyses were done as in (C). (F) The percentage of neurite-bearing cells in (E) was calculated and graphed. $^{**}p < 0.01$.

3.2. MAGI-1 interacts with p75NTR

We tried to identify possible MAGI-1-interacting partner(s) involved in NGF-mediated signaling pathway in PC12 cells. NGF is known to bind with high affinity receptor, TrkA, and low affinity receptor, p75NTR [12]. Through amino acid sequence analyses of TrkA and p75NTR using NCBI database, we found that p75NTR (NCBI accession no. NM_012610) contains a putative carboxyl-terminal type 1 PDZ binding (TSPV) motif [27]. In fact, the interaction of p75NTR and another PDZ protein, Par-3, has been reported [28]. Since MAGI-1 contains multiple PDZ domains known to mediate interaction with several molecules, we assumed that MAGI-1 interacts with p75NTR and thus tested the possibility. COS7 cells were transfected with GFP-p75NTR with or without myc-MAGI-1 and immunoprecipitation was carried out using anti-MAGI-1. As shown in Fig. 2A, complex formation of MAGI-1 with p75NTR was observed. This interaction is likely to be specific since MAGI-2 and MAGI-3 did not bind with p75NTR under the conditions (Fig. 2A). Next, when we immunoprecipitated endogenous p75NTR from extract of PC12 cells, endogenous MAGI-1 was detected in precipitates (Fig. 2B), strongly suggesting their physiological interaction. Notably, when cells were stimulated with NGF, association between p75NTR and MAGI-1 was increased (Fig. 2B). We then did mapping analyses to determine regions essential for the association between MAGI-1 and p75NTR. Various myc-tagged MAGI-1 fragments were co-expressed with GFP-p75NTR and immunoprecipitation was done using anti-GFP. As shown in Fig. 2C, PDZ0-1 was found to interact with p75NTR while PDZ1–3 and PDZ4–5 were not. We thus concluded that N-terminal part containing PDZ0, GK and WW domain is responsible for the binding to p75NTR. We then tried to determine the region in p75NTR essential for the binding with MAGI-1. COS7 cells were transfected with

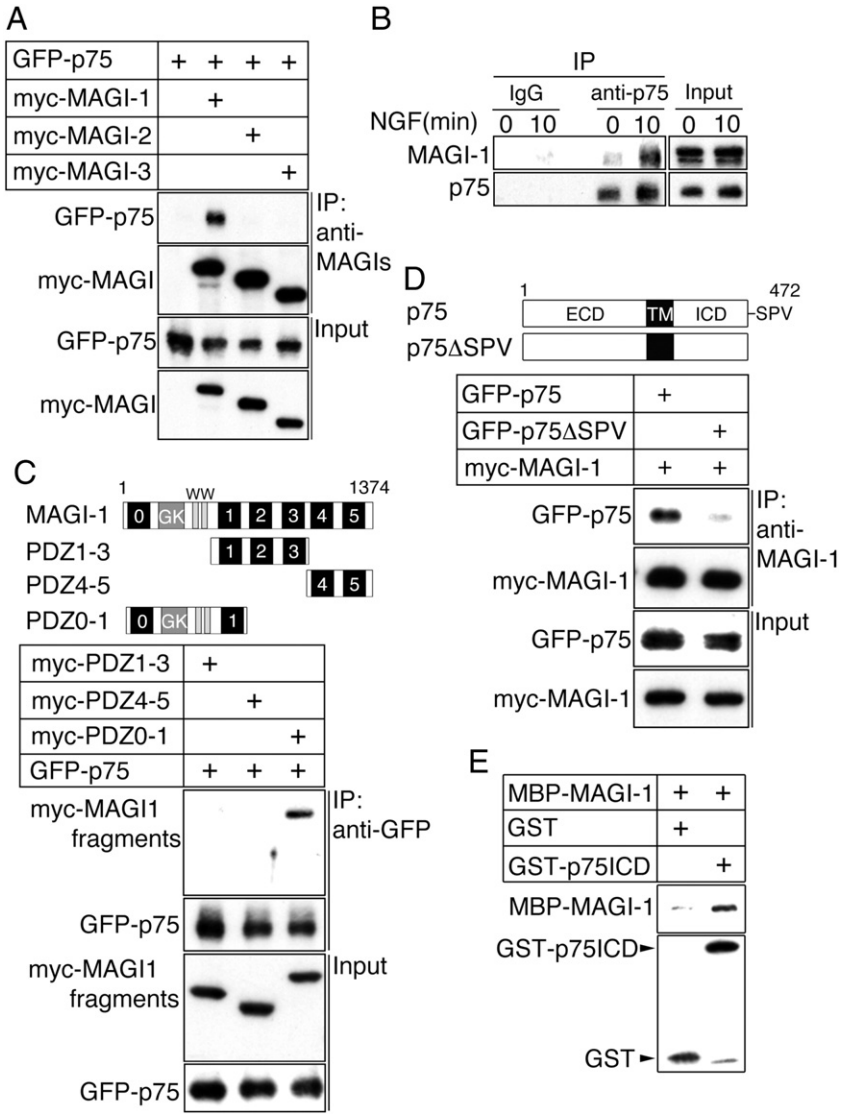

Fig. 2. MAGI-1 interacts with p75NTR. (A) COS7 cells were transfected with pEGFP-p75NTR together with pRK5 vector, pRK5-myc-MAGI-1, -myc-MAGI-2 or -myc-MAGI-3. Cell lysates were subjected to immunoprecipitation using anti-MAGI1/2/3. Western blotting of precipitated materials (IP) and lysate (Input) was done using anti-myc or anti-GFP. (B) PC12 cells were serum starved for 6 h and then stimulated with 100 ng/ml NGF for 10 min or left untreated. Cells were then harvested and p75NTR was immunoprecipitated with polyclonal anti-p75. Precipitated materials (IP) and lysates (Input) were analyzed by western blotting with anti-MAGI-1 or monoclonal anti-p75NTR. (C) COS7 cells were transfected with GFP-p75NTR together with various myc-tagged MAGI-1 fragments. Cell lysates were subjected to immunoprecipitation using anti-GFP. Precipitated materials (IP) and lysates (Input) were subjected to SDS-PAGE followed by western blotting using anti-GFP or anti-myc. (D) COS7 cells were transfected with myc-MAGI-1 in the presence of GFP-p75NTR or -p75NTRΔSPV lacking C-terminal three amino acids. Cell lysates were subjected to immunoprecipitation using anti-MAGI-1. Precipitated materials (IP) and lysates (Input) were subjected to western blotting using anti-GFP or anti-myc. (E) In vitro binding assay of MAGI-1 and p75NTR. Recombinant MBP-MAGI-1 (400 pmol) was incubated with GST (100 pmol) or GST-p75NTR intracellular domain (ICD) (100 pmol) conjugated glutathione sepharose beads. Then beads were washed and bounded materials were subjected to SDS-PAGE followed by western blotting using anti-MBP or anti-GST.

myc-MAGI-1 together with GFP-p75NTR or GFP-p75NTRΔSPV lacking the C-terminal PDZ binding motif. When immunoprecipitation was done using anti-MAGI-1, p75NTRΔSPV was shown to have dramatically reduced binding ability to MAGI-1 (Fig. 2D), indicating the importance of the C-terminal SPV motif for the interaction with MAGI-1. We next tested whether recombinant MAGI-1 directly associates with recombinant p75NTR or not. Since full length p75NTR was barely expressed in *E. coli*, we used the intracellular domain (ICD) containing the C-terminal PDZ-binding motif. Consequently, MBP-MAGI-1 was shown to associate with GST-p75NTR-ICD (Fig. 2E). Based on these results, we concluded that PDZ0 domain localized at N-terminus of MAGI-1 interacts directly with the C-terminal SPV motif of p75NTR.

We determined the localization of MAGI-1 and p75NTR in PC12 cells by immunofluorescence. In undifferentiated PC12 cells, MAGI-1 was distributed at cell body and cellular edges while p75NTR was enriched at cellular edges (Fig. 3A, upper panels). When differentiated, MAGI-1 and p75NTR were found to be distributed at the tip of extended neuronal process in addition to cytoplasm and cell peripheral areas (Fig. 3A, lower panels). These results indicate that endogenous MAGI-1 and p75NTR are, at least partly, co-localized in PC12 cells, suggestive of physiological interaction of these proteins. We then looked into the effect of MAGI-1 knockdown on p75NTR localization in PC12 cells. When MAGI-1 was silenced in the cells, and subcellular distribution of p75NTR was analyzed, significant change was not observed under the assay conditions (Fig. 3B).

3.3. MAGI-1 interacts with an adaptor protein Shc

p75NTR has been reported to enhance TrkA signaling through the interaction with an adaptor protein Shc, although it is not clear whether the interaction is direct or not [17]. Shc contains an N-terminal phosphotyrosine binding (PTB) domain, a central glycine/proline-rich (Gly/Pro) region and a C-terminal Src homology 2 (SH2) domain [29]. Since MAGI-1 contains two WW domains, which possibly interact with proline-rich motif, we examined the interaction of MAGI-1 with Shc by immunoprecipitation. COS7 cells were transfected with FLAG-Shc with or without myc-MAGI-1 and cell lysates were subjected to immunoprecipitation using anti-MAGI-1. As shown in Fig. 4A, MAGI-1 was found to form an immunocomplex with Shc. The interaction of MAGI-1 with Shc was concluded as a direct binding since recombinant MBP-MAGI-1 was pulled down with GST-Shc formed in a reconstitution assay (Fig. 4B). We then carried out mapping analyses to determine regions responsible for the association between MAGI-1 and Shc. To determine region in MAGI-1 responsible for binding to Shc, various

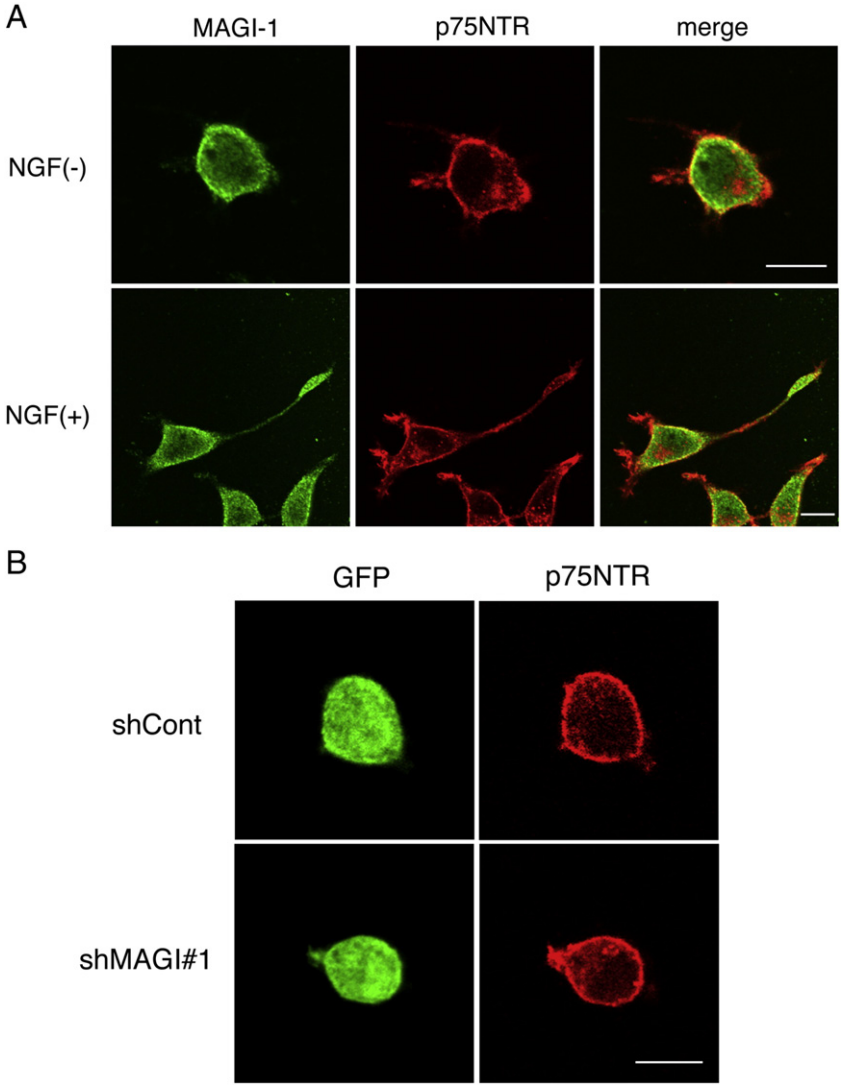

Fig. 3. Localization of MAGI-1 and p75NTR in PC12 cells. (A) Cells were cultured in the presence (+) or absence (−) of 100 ng/ml NGF for 72 h, fixed and double-stained with anti-MAGI-1 and anti-p75NTR. (B) Cells were transfected with control pSUPER-GFP or pSUPER-GFP-MAGI#1 (shMAGI#1). After 48 h, cells were fixed and stained with anti-p75NTR. Bars, 10 μm.

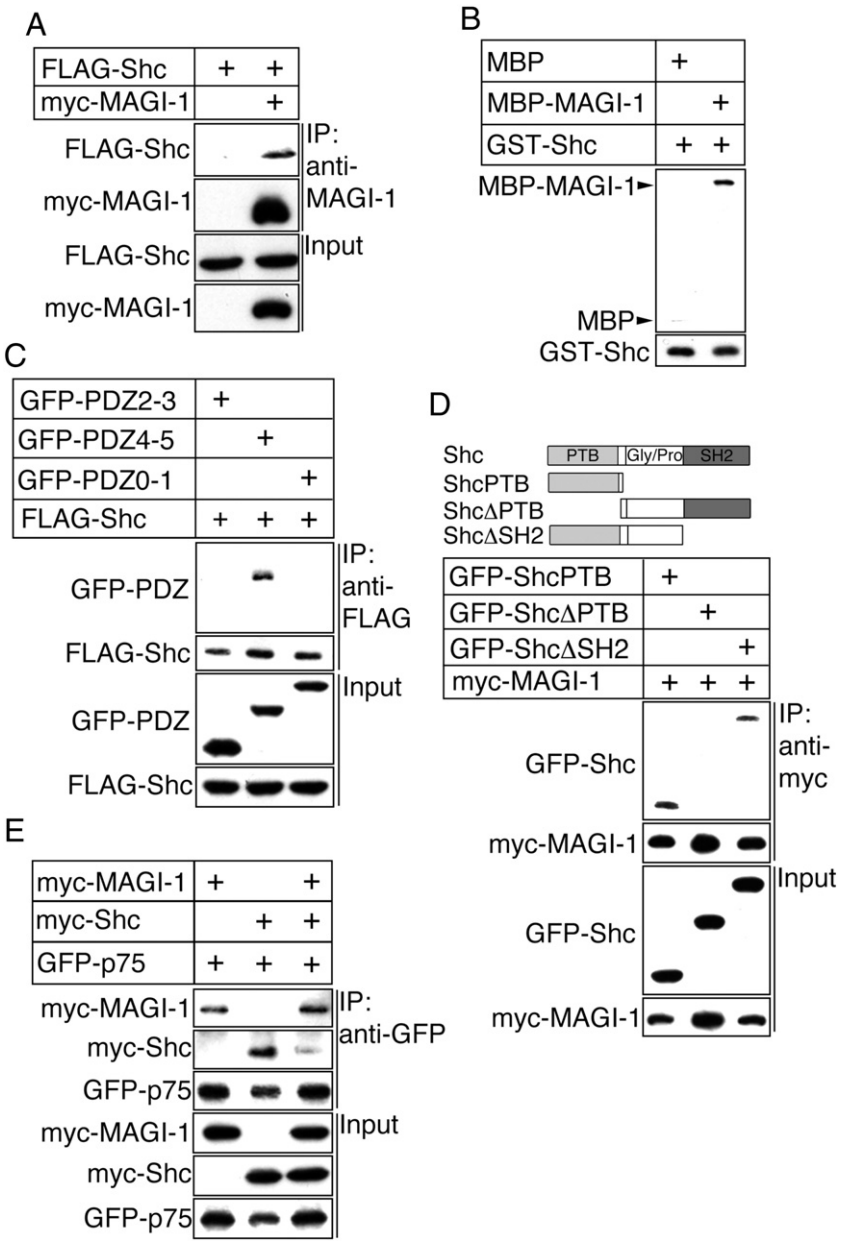

Fig. 4. MAGI-1 interacts with Shc. (A) COS7 cells were transfected with FLAG-Shc with or without myc-MAGI-1. Cell lysates were subjected to immunoprecipitation using anti-MAGI-1. Western blotting of precipitated materials (IP) and lysates (Input) was carried out with anti-myc or anti-FLAG. (B) In vitro binding assay of MAGI-1 and Shc. Recombinant MBP-MAGI-1 (200 pmol) or MBP (200 pmol) was incubated with GST-Shc (100 pmol) conjugated with glutathione sepharose beads. Then beads were washed and bound materials were subjected to SDS-PAGE followed by western blotting with anti-MBP or anti-GST. (C) COS7 cells were transfected with FLAG-Shc together with various fragments of GFP-MAGI-1. Cell lysates were subjected to immunoprecipitation using anti-FLAG. Precipitated materials (IP) and lysates (Input) were subjected to western blotting with anti-GFP or anti-FLAG. (D) COS7 cells were transfected with myc-MAGI-1 together with various GFP-tagged Shc fragments. Cell lysates were subjected to immunoprecipitation using anti-myc. Precipitated materials (IP) and lysates (Input) were subjected to western blotting with anti-myc or anti-GFP. (E) COS7 cells were transfected with myc-MAGI-1, myc-Shc and GFP-p75NTR in various combinations. Cells were harvested and p75NTR was immunoprecipitated with polyclonal anti-p75. Precipitated materials (IP) and lysates (Input) were subjected to western blotting with anti-GFP or anti-myc.

GFP-tagged MAGI-1 fragments were co-transfected with Flag-Shc and immunoprecipitation was done using anti-FLAG. As shown in Fig. 4C, PDZ4-5 in MAGI-1 was co-immunoprecipitated with Shc. Next, to determine binding region in Shc, we transfected various GFP-tagged Shc fragments with myc-MAGI-1 and immunoprecipitation was done using anti-myc. Consequently, ShcPTB and ShcΔSH2 but not ShcΔPTB were co-immunoprecipitated with MAGI-1 (Fig. 4D), indicating the PTB region is responsible for the binding to MAGI-1. From these results, we concluded that PDZ4-5 in MAGI-1 interacts directly with PTB region in Shc.

Since MAGI-1 was found to interact with p75NTR and Shc, we here asked if these three molecules form a ternary complex or not. When myc-MAGI-1, myc-Shc and GFP-p75NTR were expressed in COS7 cells and GFP-p75NTR was immunoprecipitated by anti-p75, the three molecules were found to form a ternary complex (Fig. 4E).

3.4. MAGI-1 regulates NGF-dependent phosphorylation of Shc and ERK in PC12 cells

NGF binds to the TrkA/p75NTR receptor complex and stimulates tyrosine phosphorylation of Shc, although molecular mechanism of the receptor-Shc interaction has been enigmatic. MAGI-1 interacts directly with p75NTR and Shc, indicating that MAGI-1 functions as a linker

between p75NTR and Shc. This observation suggests involvement of MAGI-1 in NGF-stimulated signaling pathway leading to the subsequent activation of Shc/Ras/Raf/ERK pathway. Thus, we examined the role of MAGI-1 in the NGF-dependent activation of Shc and ERK in PC12 cells by the use of RNAi technique. As mentioned above, transfection efficiency of PC12 cells was relatively low, we could not observed the effect of MAGI-1 knockdown on the NGF-induced activation of endogenous Shc and ERK (data not shown). To monitor the effect of MAGI-1 silencing on the activation of Shc and ERK, we did co-transfection of MAGI-1 knockdown vector and GFP-Shc or GFP-ERK expression vector. GFP-Shc and GFP-ERK were distinguished from endogenous molecules by western blotting because molecular masses of these proteins were different. Trancfected PC12 cells were stimulated with NGF for 5 min or 30 min and phosphorylated (activated) GFP-Shc was detected by western blotting using anti-phospho Shc. We found that the phosphorylation was significantly suppressed in MAGI-1-deficient cells (Fig. 5A and B). Using similar technique, we also found that knockdown of MAGI-1 caused the suppression of ERK activity after NGF treatment (Fig. 5C and D). These results suggest that MAGI-1 is involved in the positive regulation of NGF-stimulated Shc-ERK pathway. NGF-induced phosphorylation of GFP-Shc and -ERK was not completely suppressed in the above experiments. Since MAGI-2 and MAGI-3 do not interact with p75NTR (Fig. 2A), we assume that this residual phosphorylation was due to the incomplete knockdown efficiency of the pSUPER-MAGI-1 RNAi vector although the possibility of yet unidentified signaling pathway(s) cannot be ruled out.

4. Discussion

Biological information of individual MAGI family proteins has been limited because of their structural similarities. We have recently developed the antibody that selectively detect MAGI-1 protein and found that MAGI-1 was enriched in rat nervous tissues [11]. Histological study of rat tissues revealed abundant expression of MAGI-1 in glomeruli of adult olfactory bulb and embryonic dorsal root entry zone in spinal cord [11]. We also found that MAGI-1 existed in the growth cone of primary cultured rat dorsal root ganglia cells [11]. From these results, we supposed that MAGI-1 might play pivotal roles in neurite extension or guidance. To ascertain this hypothesis, we did knockdown of MAGI-1 in PC12 cells, a widely used cell line for analysis of neurite outgrowth, and found that silencing of MAGI-1 caused inhibition of NGF-dependent neurite extension. On the other hand, overexpression of MAGI-1 also inhibited NGF-mediated neurite extension. This may be the reason that we could not obtain statistically clear results in the rescue experiments; it may be difficult to express proper amount of RNAi-resistant MAGI-1 which exactly compensates the RNAi-mediated loss of endogenous MAGI-1. We consider that this is the first report describing the function of MAGI-1 in neural development.

Trk and p75NTR synergistically affect the downstream signaling cascades that lead to the neurite extension [12]. It has been reported that p75NTR specifically enhanced phosphorylation of Shc during NGF-induced TrkA activation but the mode of interaction between TrkA/p75NTR and Shc has not been clarified [17]. In this study, we

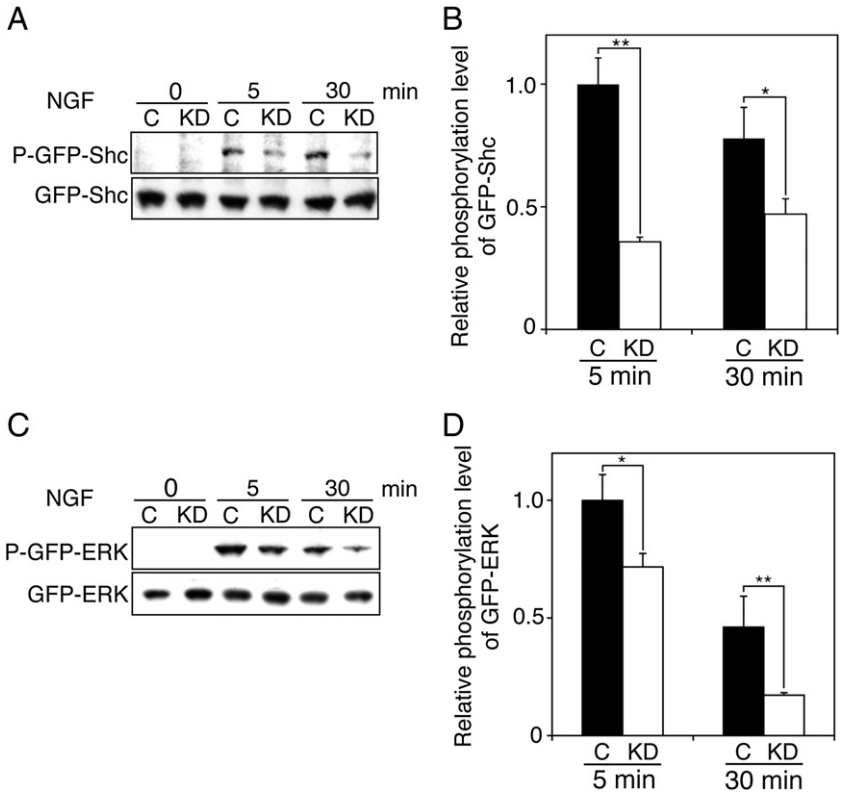

Fig. 5. MAGI-1 is involved in the NGF-dependent signaling pathway in PC12 cells. (A) Cells were transfected with GFP-Shc in the presence of control (*C*) or pSUPER-MAGI-1#1 vector (*KD*), cultured for 48 h and then serum-starved for 24 h. Cells were then stimulated with 100 ng/ml NGF for indicated time and harvested. Whole cell lysates were subjected to SDS-PAGE followed by western blotting using anti-phospho-Shc or anti-GFP. (B) Relative levels of phosphorylated Shc in lysates were quantified with ImagePro PLUS software based on densitometry, and expressed as relative intensity to the band of 5 min-stimulated pSUPER transfected control cells. Because band intensities of phosphorylated Shc before NGF stimulation (time 0) were less than detection limit, we showed data of 5 min and 30 min-stimulated cells. Each bar shows the mean ± SD of results from 4 dishes. The data shown are representatives of 3 independent experiments with similar results. $^{*}p < 0.05$. (C) Cells were transfected with GFP-ERK together with control (*C*) or pSUPER-MAGI-1#1 vector (*KD*), cultured for 48 h and serum-starved for 24 h. Cells were then stimulated with 100 ng/ml NGF for indicated time and harvested. Lysates were subjected to western blotting with anti-phospho-ERK or anti-GFP. (D) Relative levels of activated ERK in lysates were calculated with ImagePro PLUS software based on densitometry, and expressed as relative intensity to the band of pSUPER transfected control cells treated with NGF for 5 min. Each bar shows the mean ± SD of results from 4 dishes. The data shown are representatives of 3 independent experiments with similar results. $^{*}p < 0.05$, $^{**}p < 0.01$.

found that MAGI-1 interacted directly with not only p75NTR but also Shc, both of which are principal components in NGF-mediated signaling. From mapping analysis, p75NTR and Shc bind different regions in MAGI-1 molecule, supporting the notion that these three proteins may form a ternary complex.

We have hypothesized that WW domain in MAGI-1 could form complex with glycine/proline-rich domain in Shc. Contrary to our assumption, PDZ4–5 region in MAGI-1 could interact with PTB region in Shc. PTB domain is known to interact with NPX(p)Y-like motif (where pY is phosphotyrosine) [30]. We tried to find NPX(p)Y-like motif in PDZ4–5 region in MAGI-1 using GPS2.1, a software that predict kinase-specific phosphorylation site. However, we could not find the PTB binding motif in that region (data not shown). Recently, Chen et al. reported that a PTB domain containing protein tensin2 could interact with deleted in liver cancer 1 (DLC1), which does not have NPX(p)Y-like motif and proposed a novel binding mode of PTB domain [31]. Such a novel binding mechanism may be involved in the interaction of MAGI-1 with Shc although additional extensive experiments are required to understand the binding mode of these molecules.

In PC12 cells, NGF induces transient (<5 min) and sustained (>30 min) activation of ERK and the latter is considered to play an essential role for neurite extension [32]. There are several ERK activating pathways that lead to the neurite extension. NGF-induced activation of FRS2/Crk/C3G/Rap1/B-Raf pathway mediated the sustained activation of ERK [32]. In contrast, other group has reported that a very little contribution of NGF-induced Rap1 activation on ERK activity [33]. Regulators of G-protein signaling (RGS) 12 has been reported to act as a Ras/B-Raf/MEK scaffold and play a critical role in NGF-mediated differentiation [34]. Dual-specificity tyrosine-phosphorylated and regulated kinase 1A (DYRK1A) facilitates the formation of a Ras/B-Raf/MEK-1 complex and upregulates ERK activation [35]. In this study, we showed that NGF-induced transient and sustained activation of Shc and ERK were suppressed by MAGI-1 silencing. Together with the results that p75NTR binds specifically with MAGI-1 but not MAGI-2 and MAGI-3, MAGI-1 is suggested to regulate neurite outgrowth through linking p75NTR and Shc-ERK pathway (Fig. 6). However, the possibility cannot be ruled out that residual phosphorylation of Shc and ERK observed in NGF-treated MAGI-1-deficient PC-12 cells (Fig. 5) is mediated by yet unidentified molecule(s).

The four neurotrophins are known to interact specifically with three members of Trk receptors, TrkA, TrkB and TrkC. NGF interacts with TrkA. While BDNF and NT-4 interact with TrkB, NT-3 interacts with TrkC. On the other hand, the neurotrophins interact with p75NTR with similar affinity [12]. We found the interaction of MAGI-1 and p75NTR that resulted to control NGF-dependent signaling pathway. It is susceptible that MAGI-1 may control the signaling pathway activated by all four neurotrophins.

Recently, genetic associations of MAGI-1 and psychiatric disorder such as bipolar disorder and schizophrenia have been reported [36,37]. The missense mutation of p75NTR (S205L) has been shown to be associated with depressive disorder and suicidal behavior [38]. The roles of neurotrophins on the neuronal development such as neurite outgrowth and synaptogenesis have been investigated extensively [14,39]. Neurodevelopmental theory of mental illness such as schizophrenia is widely accepted [40]. Regulation of the neurotrophin signaling by MAGI-1 may contribute to the pathogenesis of mental illness. Further analysis of MAGI-1 in neuronal signaling may lead to the understanding of the etiology of psychiatric disorders.

5. Conclusion

MAGI-1 interacts with p75NTR and Shc, controls NGF-stimulated activation of the Shc-ERK pathway and regulates neurite extension of PC12 cells.

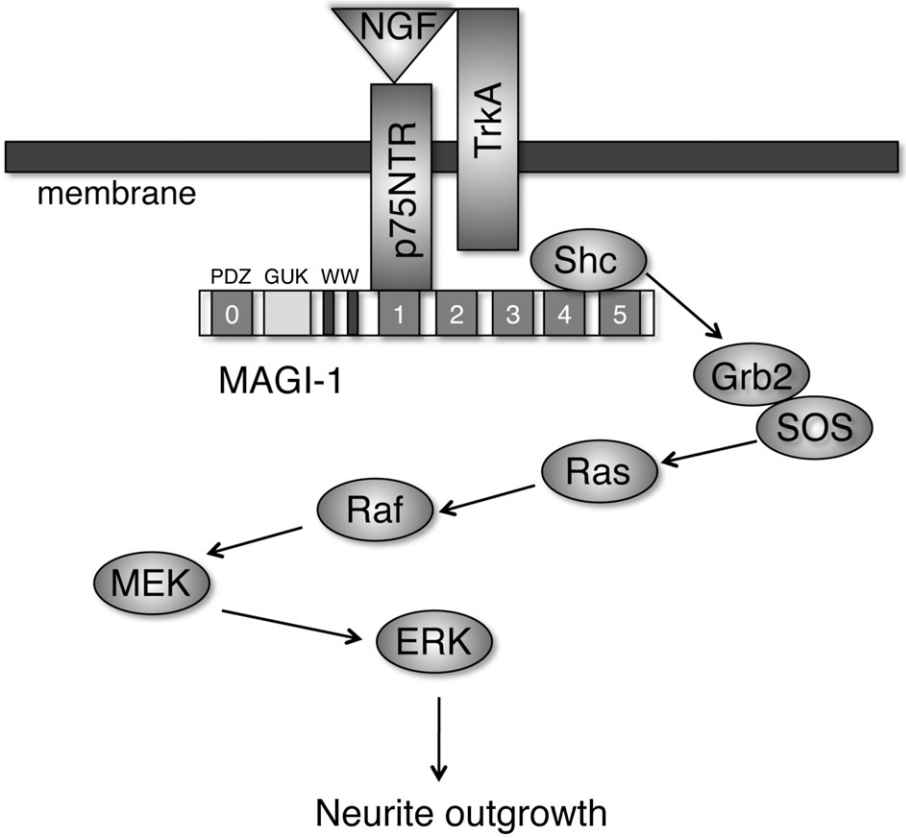

Fig. 6. Hypothetical model of MAGI-1-mediated NGF signaling. MAGI-1 localizes at juxtamembrane region in PC12 cells and acts as a scaffold for p75NTR and Shc. Upon stimulation by NGF, MAGI-1 may control the phosphorylation of Shc by TrkA and regulate the subsequent signaling pathway that induces neurite outgrowth.

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